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Elicitor-Induced Spruce Stress Lignin[1]

Structural Similarity to Early Developmental Lignins

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Suspension cultures of *Picea abies* (L.) Karst released polymeric material into the culture medium when treated with an elicitor preparation from the spruce needle pathogen *Rhizosphaera kalkhoffii*. The presence of lignin (about 35%, w/w) was demonstrated by phloroglucinol/HCl reactivity and quantitation with thioglycolic acid. Carbohydrate (about 14%, w/w) and protein (about 32%, w/w) were also detected. Amino acid analysis revealed that hydroxyproline and proline predominated. Thioacidolysis and subsequent Raney nickel desulfurization allowed the analysis of lignin-building units and interunit bonds. Compared with spruce wood lignin, an approximately 20-fold higher relative amount of *p*-hydroxyphenyl units was determined. A high content of *p*-hydroxyphenyl units is typical for certain developmental lignins, such as conifer compression wood and middle lamella lignins, as well as all induced cell culture lignins so far analyzed. Cross-linkages of the pinoresinol type (β-β) in the excreted cell culture lignin were markedly increased, whereas β-1 interunit linkages were decreased relative to spruce wood lignin. The amount and nature of cross-linkages were shown to be intermediate between those in wood lignin and in enzymatically prepared lignins. In summary, the elicitor-induced stress lignin was excreted as a lignin-extensin complex that closely resembled early developmental lignins.

Plant lignins are deposited mainly in secondary walls of lignifying tissues, especially in wood or xylem vessels, thus providing rigidity and structural support to cell wall polysaccharides (Sarkanen and Hergert, 1971). The monomeric composition of lignins varies between plant species and during plant development. Normal gymnosperm wood lignin consists predominantly (98–99%) of G units, originating from coniferyl alcohol. Angiosperm lignins are mainly a mixture of G and S units, the latter being derived from sinapyl alcohol. In grass lignins, *p*-coumaryl alcohol serves as precursor for H units, which are found in addition to S and G units. The various phenylpropanoid monomers are interconnected by ether and carbon-carbon linkages in complex bonding patterns (Freudenberg, 1968; Sakakibara, 1980). The metabolic inertness of lignin is thought to result from these complex cross-linkages and from the hydrophobic nature of lignins.

The polymerization of the lignin monomer precursors is initiated by peroxidases and/or laccases and is thought to proceed as a spontaneous process without any enzymatic control (Freudenberg, 1968; Sedoroff et al., 1994). Nevertheless, there are specific developmental lignins that differ from the general lignin structure of a given plant species. Lignin variability (Monties, 1989) may be of high significance for biological interactions and for soil humification processes. Induced lignification is one of several plant defense responses to wounding and to viral or microbial attack (Vance et al., 1980; Friend, 1981; Aist, 1983; Hargreaves and Keon, 1986; Bolwell, 1988; Stone, 1989). The structure of the induced lignins has not been well characterized but appears to differ from that of the constitutive lignin of the respective plant species (Asada and Matsumoto, 1972; Ride, 1975; Hammerschmidt et al., 1985; Doster and Bostock, 1988; Robertsen and Svalheim, 1990). We have now performed a detailed analysis of an elicitor-induced stress lignin excreted by cultured spruce (*Picea abies* [L.]) cells.

Photomixotrophic suspension cultures of *P. abies* (L.) Karst have previously been shown to release a lignin-like material into the culture medium when treated with an elicitor preparation from the spruce needle pathogen *Rhizosphaera kalkhoffii* (Meßner and Boll, 1993). Extracellular lignin induced in spruce cell cultures by a changed growth medium and without use of elicitor (Simola et al., 1992) has already been chemically characterized (Brunow et al., 1990, 1993). The elicitor-dependent ECL has been analyzed by the recently developed method of thioacidolysis (Lapierre et al., 1986, 1991). The lignin composition found is compared with those of SWL, as well as with spruce seedling and enzymatic lignins. Stress lignin is shown to resemble certain "normal" developmental lignins of spruce, in particular those of compression wood, the cell-wall middle lamella, and unelicited cell culture lignins.

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Abbreviations: CAD, cinnamyl alcohol dehydrogenase; DHP, dehydrogenation polymer; ECL, extracellular lignin; G, guaiacyl; H, *p*-hydroxyphenyl; LTGA, lignin thioglycolic acid derivatives; S, syringyl; SWL, in situ spruce wood lignin.

MATERIALS AND METHODS

Cell Culture

Photomixotrophic cell-suspension cultures of Norway spruce (*Picea abies* [L.] Karst) had been established from needles of aseptically grown seedlings (Meßner and Boll, 1993). The cultures were maintained in Murashige-Skoog basal medium (Murashige and Skoog, 1962), containing 3% (w/v) Suc, naphthylacetic acid (16.1 μM), and benzylaminopurine (4.4 μM), pH 5.7. Fernbach flasks (2 L) containing 350 mL of sterilized medium were inoculated with 35 g of cells (wet weight) and grown at 27 ± 1°C in continuous light (Osram L 40 W/77, 1000 Lux) on a reciprocal shaker at 90 rpm (Meßner and Boll, 1993). Alternatively, 200-mL Erlenmeyer flasks were used on a 10-fold smaller scale. Cultures were transferred to fresh medium every 3 to 4 d. Growth was monitored by measuring cell fresh weight and conductivity of the medium.

Elicitor Treatment

Rhizosphaera kalkhoffii was cultivated and a crude elicitor fraction was prepared as described by Meßner and Boll (1993). Three-day-old Norway spruce cell cultures (350 mL) were incubated with 10 mL of elicitor suspension per culture flask (corresponding to 1.0 mg of Glc equivalents per mL culture medium; neutral sugar content determined according to the method of Ayers et al., 1976). To controls, 10 mL of water were added. Cells were harvested by filtration onto filter paper (MN 615; Macherey and Nagel, Düren, Germany), thoroughly rinsed with deionized water, weighed, shock-frozen in liquid nitrogen, and stored at −20°C until further analyzed.

Isolation of the Extracellular Polymer

Polymer-containing culture medium was passed through nylon tissue to remove the cultured spruce cells. The filtrate was centrifuged for 30 min at 24,000*g*, and the supernatant was rejected. The pellet material was treated consecutively with 15 mL of the following solvents and solutions with mixing for 15 min, followed by centrifugation as above: (a) 1 M NaCl, (b) 1% (w/v) SDS, and (c) H_2O (three times). The purified polymer was freeze-dried, homogenized in a mortar, and stored at −20°C until analysis. The yield of polymeric material varied between 20 and 25 mg/350 mL/medium at d 7 postelicitation.

Plant Material

Seeds of *P. abies* (L.) Karst (year of maturation 1985; provenance No. 84019; Staatliche Samenklenge Laufen, Laufen, Germany) were germinated, and seedlings were grown on humid Perlite in a controlled environment chamber at 25/20 ± 1°C (day/night), 16-h photoperiod (6–22 h), 100 $\mu E\ s^{-1}\ m^{-2}$ (50 $W\ m^{-2}$) light intensity, and 70 ± 5% RH (Langebartels et al., 1991). Seedlings were harvested 6 weeks after sowing.

Cell-Wall Preparation

Aliquots (approximately 300 mg) of frozen suspension-cultured cells, as well as needle or hypocotyl tissue from spruce seedlings, were ground in a mortar in the presence of liquid nitrogen. The resulting fine powder was suspended in 1.5 mL of methanol and transferred to a 2-mL Eppendorf vial. The mixture was vigorously stirred for 1 h and centrifuged (5 min in a bench-top centrifuge). The pellet was consecutively treated with 1.5 mL of the following solvents and solutions with mixing for 15 min, followed by centrifugation for 5 min as above: (a) methanol (twice), (b) 1 M NaCl, (c) 1% (w/v) SDS, (d) H_2O (twice), (e) ethanol, (f) $CHCl_3/CH_3OH$ (1:1, v/v), and (g) *tert*-butyl methyl ether. The remaining insoluble material (purified cell walls) was freeze-dried overnight.

Quantitation of Lignin

Lignin was assayed by derivatization with thioglycolic acid (modified from the method of Bruce and West, 1989; Bonello et al., 1993). Approximately 10 to 15 mg of the purified extracellular polymer or cell-wall preparations were placed in a 1.5-mL Eppendorf screw-cap vial and treated with 1 mL of 2 M HCl and 0.2 mL of thioglycolic acid for 4 h at 95°C. After cooling to room temperature, the mixture was centrifuged for 10 min in a bench-top centrifuge. The supernatant was removed with a Pasteur pipette, and the remaining pellet was washed three times with H_2O. The pellet was suspended in 1 mL of 0.5 M NaOH and vigorously shaken overnight to extract the LTGA. Following centrifugation as above, the supernatant was decanted into a 2-mL Eppendorf vial, and the pellet was washed with 0.5 mL of 0.5 M NaOH. The combined alkali extract was acidified with 0.3 mL of concentrated HCl and the LTGA was allowed to precipitate at 4°C for 4 h. The mixture was centrifuged as above, the supernatant was removed with a Pasteur pipette, and the brown pellets were dried in a SpeedVac centrifuge. The pellet was dissolved in 1 mL of 0.5 M NaOH and diluted to 40 mL with 0.5 M NaOH. Absorption at 280 nm was then measured. A calibration curve was obtained using a DHP, which was prepared by peroxidase polymerization of coniferyl alcohol. An absorption value of 0.316 per mg initial lignin was obtained.

Spectroscopic Methods

Samples were acetylated for ^{1}H-NMR analysis to improve the spectral dispersion of signals in the ^{1}H-NMR spectra (according to the method of Brunow et al., 1990). Spectra were recorded at 303°K with an AC 400 NMR spectrometer (Bruker, Karlsruhe, Germany) using d_6-DMSO as a solvent. For Fourier transform IR analysis samples were embedded in a KBr pellet (5%; w/w), and the spectra were recorded between 370 and 4000 cm^{-1} (system 2000 Fourier transform-IR spectrometer, Perkin-Elmer).

Thioacidolysis

The thioacidolysis reagent was prepared by mixing 2.5 mL of BF_3 etherate and 10 mL of ethanethiol and adjusting

the final volume to 100 mL with dioxane. The final reagent consisted of 0.2 M BF_3 etherate in dioxane/ethanethiol, 8.75:1 (v/v).

The sample (approximately 5 mg) was added to 5 mL of the thioacidolysis reagent in a glass tube closed with a Teflon-lined screwcap. Thioacidolysis was performed at 100°C for 4 h in an oil bath with occasional shaking. The ice-cooled reaction mixture (as well as 3 × 10 mL of H_2O used to rinse the tube) was then poured into 30 mL of CH_2Cl_2 containing 0.25 mg of docosane, the GC internal standard. The pH of the aqueous phase was adjusted to 3 to 4 by addition of 0.4 M $NaHCO_3$. After the aqueous phase was repeatedly extracted with CH_2Cl_2, the combined organic extracts were dried over Na_2SO_4. Solvent was removed under reduced pressure at 40°C. The residue was redissolved in 1 mL of CH_2Cl_2. An aliquot (approximately 10 μL) was silylated with 50 μL of bistrimethylsilyltrifluoro-acetamide and 10 μL of pyridine in a 200-μL silylation vial fitted with a Teflon-lined screw cap. Silylation was complete after 30 min.

Desulfurization

An aliquot of the thioacidolysis products (approximately 800 μL of the CH_2Cl_2 solution) was placed in a screw-cap glass tube containing 1 to 2 mL of Raney nickel aqueous slurry (Aldrich) and 5 mL of dioxane. The reaction was performed at 50°C for 4 h in an oil bath. The ice-cooled reaction mixture was poured into 30 mL of CH_2Cl_2 and the tube was rinsed with 10 to 20 mL of H_2O. The combined aqueous layer was extracted three more times with CH_2Cl_2. Raney nickel remained in the aqueous phase. The combined organic extracts were dried over Na_2SO_4, and the solvent was removed in vacuo. The residue was redissolved in 0.2 mL of CH_2Cl_2, and silylation was performed as described above.

Analysis of Reaction Products

GC analysis was performed using a gas chromatograph equipped with a moving-needle-type injector and a flame ionization detector. Silylated samples (1–6 μL) were injected onto a polydimethylsiloxane capillary column (DB 1, 30 m × 0.32 mm i.d., film thickness 0.25 μm; Supelco, Belafonte, PA) and eluted with helium as the carrier gas (inlet pressure 1.2 bar) using a temperature program from 160 to 260°C at 2°C min^{-1}. Mass spectra were recorded at 70 eV (ion source at 160°C) with a Nermag R 10–10H quadrupole mass spectrometer combined with a Delsi DI 700 gas chromatograph with the same column as above and a temperature program from 110 to 250°C at 3°C min^{-1}. The inlet pressure of helium was 0.6 bar. Lignin degradation products were identified by comparison with the previously described reference spectra (Lapierre et al., 1986, 1991).

Amino Acid and Sugar Analysis

Protein was hydrolyzed at 105°C in constantly boiling 6 M HCl for 24 h. Under these conditions, Trp, Cys, and Met were decomposed. After HCl was removed in vacuo, the hydrolysate was dissolved in aqueous buffer and derivatized with phenylisothiocyanate by a standard procedure (Derivatizer EBI 420 A, Applied Biosystems). The resulting phenylthiocarbamoyl amino acids were separated by HPLC (Eulitz et al., 1991). Protease digestion was performed using pronase E (from *Streptomyces griseus*), trypsin or chymotrypsin (both from bovine pancreas), which were purchased from Boehringer Mannheim. For protein digestion of the extracellular polymer from spruce cell cultures, 500 units of the respective protease were used in Tris/HCl buffer (50 mM, pH 7.5) at 30°C for 48 h. Neutral sugar content was estimated as Glc equivalents by the anthrone method (Ayers et al., 1976).

Preparation of Enzymatic Lignin

Horseradish peroxidase (500 units, EC 1.11.1.7; Boehringer Mannheim; Catalog No. 413470) was dissolved in 75 mL of 0.1 M potassium phosphate buffer (pH 6.5) and placed in a round-bottom flask that was equipped with three inlet tubes, a magnetic stirring bar, and a nitrogen inlet. Separate solutions (same buffer) of coniferyl alcohol (1.11 mmol, 150 mL), *p*-coumaryl alcohol (0.2 mmol, 150 mL), and H_2O_2 (1.40 mmol, 150 mL) were added simultaneously via a multichannel peristaltic pump (Ismatec, Wertheim, Germany) at a rate of 8 mL/h each. All solutions were kept in the dark under nitrogen. After 24 h, the polymer-containing reaction mixture was centrifuged for 30 min at 24,000*g* and the supernatant rejected. The polymeric product was treated consecutively with 15 mL of the following solvents and solutions with mixing for 15 min, followed by centrifugation as above: (a) 1 M NaCl, (b) 1% (w/v) SDS, and (c) H_2O (three times). The purified polymer was freeze-dried, homogenized in a mortar, and stored at −20°C until analysis.

RESULTS

Lignin Induction

Incubation of *P. abies* suspension cultures with an elicitor preparation from the spruce needle pathogen *R. kalkhoffii* on d 3 after subcultivation resulted in a rapid clouding of the culture medium. Staining with phloroglucinol/HCl revealed the presence of lignin-like polymers in the cell walls and the culture medium in only elicitor-treated cultures (Meßner and Boll, 1993). Staining for lipid-polyester components with Sudan Black B failed, indicating that lignin, and not suberin, was rapidly synthesized in the elicited cultures. The solubility of ECL as determined by derivatization with thioglycolic acid (LTGA assay) was as follows: 26% in dimethylformamide, 23% in DMSO, 20% in 0.1 M NaOH, 17% in 1,4-dioxane, <5% in tetrahydrofurane, and <5% in chloroform.

The induction kinetics of lignin-like material in cell walls and in the culture medium of spruce cell cultures was followed by the LTGA assay (Fig. 1) ECL was first measur-

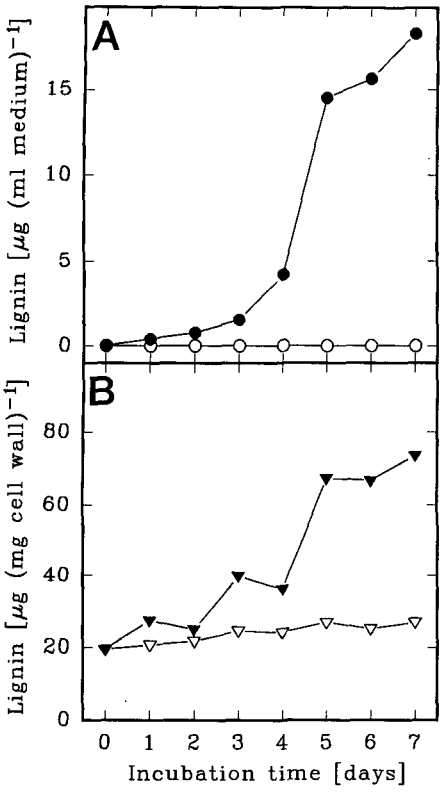

Figure 1. Time course of lignin accumulation as measured by the thioglycolic acid assay in the medium (A) and in the cell walls (B) of elicitor-treated spruce cell cultures (350 mL) after treatment with 10 mL (corresponding to 1.0 mg Glc equivalents per mL culture medium) of a *R. kalkhoffii* elicitor. To control cultures, 20 mL of water were added. The elicitor was added to 3-d-old cultures. This time is plotted as incubation time zero. Two parallel cultures and two control cultures were analyzed. Mean values are plotted. Open symbols, Control cultures. Filled symbols, Elicitor-treated cultures.

able 1 d after elicitor addition (0.4 μg lignin/mL culture medium). There was a slight increase until d 3 and a more rapid increase until d 5, followed by a slow increase until the end of the experiment (18–20 μg lignin/mL culture medium). No lignin was detected in the culture medium of control cultures. Cell walls from control cultures contained constitutive amounts of lignin (19 μg lignin/mg cell-wall material). Following elicitor treatment, cell-wall lignin accumulated to 74 μg/mg cell wall at the end of the experimental period. The lignin content of ECL harvested 5 d after elicitor addition amounted to 35% (w/w) as determined by the LTGA assay. The amount of ECL per 35-mL flask was about 700 μg at d 7. An approximately 50-fold higher amount of lignin (35 mg) was associated with the cells (8 g wet weight). On the other hand, cell-wall lignin was induced only 4-fold from a certain constitutive level, whereas ECL induction proceeded from a zero level (see Fig. 1). Further studies concentrated on ECL, because a similar lignin material had previously been chemically characterized from an independent spruce cell culture (Brunow et al., 1990, 1993, Simola et al., 1992). The previously described growth conditions optimal for lignin formation (Simola et al., 1992) also induced lignin formation without use of elicitor in the present culture (H. Glatzel, unpublished results). In addition, elicitor preparations from several mycorrhizal fungi, and even from bakers' yeast, were as active as the elicitor preparations from needle pathogens (Glatzel et al., 1993; G. Bahnweg, unpublished results).

Analysis of Lignin-Building Units

The nature and relative frequencies of the main building units and interunit bonds in the excreted polymer were determined by thioacidolysis (Lapierre et al., 1986) using BF_3 etherate in ethanethiol-dioxane as the depolymerizing reagent. The reaction mixture was analyzed after silylation by GC-MS. The main monomeric structures obtained after thioacidolysis are shown in Figure 2.

The amount of the predominant G diastereoisomer **1** and of the minor analog **2** reflect the content of lignin G units involved only in β-*O*-4 bondings. Compound **3** is formed in analogy to **1** from H units. The β-*O*-4-linked coniferyl aldehyde and coniferyl alcohol end groups yield the products **4** and **5** + **6**, respectively. The analytical results are summarized in Table I.

The absolute thioacidolysis yield was substantially lower in ECL than in SWL or in DHP (291 versus 950 and 630 μmol/g lignin, respectively). In contrast, the relative yield of H monomer **3**, compared to G monomer **1**, was remarkably higher in ECL (19%) than in SWL (1%). The figure for ECL was comparable to the one obtained for compression wood lignin (20%; Lapierre, 1993) and for the DHP sample (17%, Table I). The latter value was almost identical with the initial percentage of H precursor co-polymerized with the G precursor (18%). The relative proportion of coniferyl alcohol end groups (**5** and **6**) was significantly higher in ECL (16% of the G monomer **1**) than in SWL (7%) but lower than in DHP (36%). No *p*-coumaric acid, ferulic acid, or tyramine could be detected after alkaline or acid hydrolysis of ECL, as followed by diode array/HPLC analysis.

Lignin Interunit Linkages

The GC analysis of dimeric structures from thioacidolysis was performed after treating the product mixture with Raney nickel to desulfurize the thioethylated derivatives. This procedure allows the determination of lignin-derived dimers that have retained the resistant carbon-carbon or diaryl ether (4-*O*-5) bonds. The structures of the main products are shown in Figure 3. The dimers (**7** and **8, 9** and **10, 11** and **12, 13, 14**) correspond to the main lignin-bonding types, namely biphenyl (5–5), β-5, pinoresinol (β-β), β-1, and diaryl ether (4-*O*-5), respectively. Their relative amounts shown in Table I thus reflect the relative importance of the corresponding interunit linkages in the polymer. In comparison to SWL, ECL contained a much higher relative amount of dimers derived from pinoresinol (β-β) units (traces versus 25%). Degradation products from the β-1 interunit bonds of ECL reached only 30% of the relative concentration in SWL. Similar results were obtained with DHP. The relative yields of products from β-5, 5–5, and 4-*O*-5 units in ECL were on the same level as in SWL. DHP

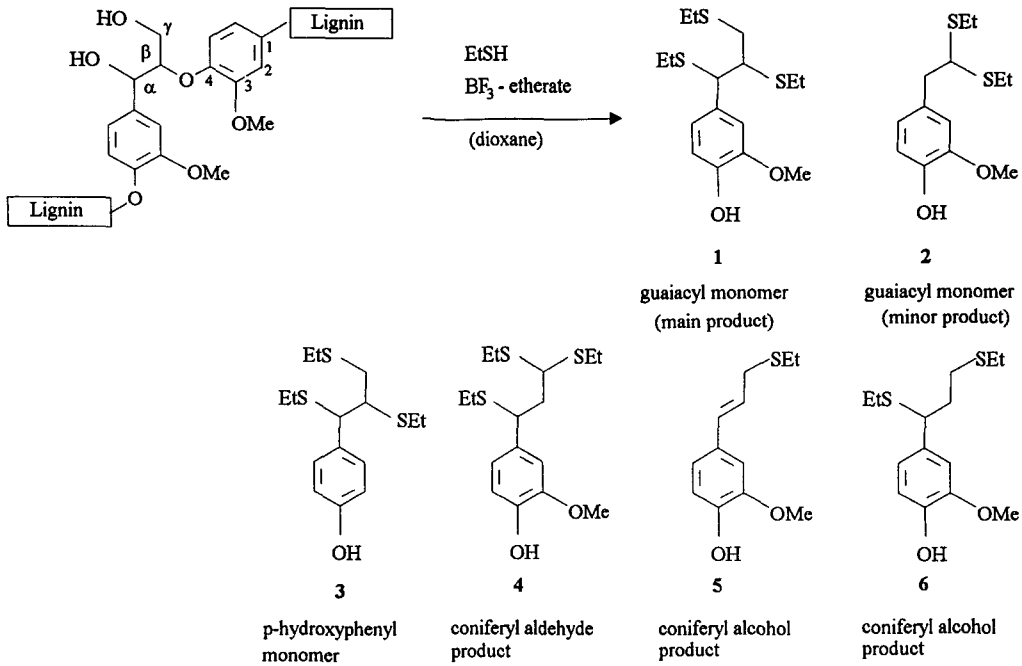

Figure 2. Chemical structures of the main monomer lignin components obtained by thioacidolysis. Compounds **1** and **2** originate mainly from the outlined lignin structure, whereas the other compounds stem from lignin structures with different ring (**3**) or side-chain functionalities (**4–6**). OMe, methoxy; EtSH, thioethyl; EtS, thioethyl.

contained more β-5 interunit linkages than ECL and SWL. Small amounts of H-G dimers (8% of G-G dimers) were detected in ECL and DHP, among which products from β-β and 5–5 interunit linkages were most abundant. The relative yield of dimeric thioacidolysis products from ECL (32% relative to the main monomeric G product, **1**) was higher than from SWL (29%) and comparable to that from DHP (36%). These data show a more condensed nature of ECL and DHP relative to SWL.

Spectroscopic Characterization

The spectroscopic properties of ECL, which was acetylated according to the method of Brunow et al. (1993), were studied by ^{1}H NMR spectroscopy. Because of the low solubility of acetylated ECL in common solvents such as acetone, chloroform, or pyridine, d_6-dimethylsulfoxide was chosen for NMR spectroscopy. The most prominent signals were assigned to aliphatic acetate protons (2.0 ppm), aro-

Table I. *Monomeric and dimeric product yields obtained after thioacidolysis of the ECL-like material from spruce cell cultures in comparison with SWL and a DHP*

Values are expressed as μmol/g lignin (ECL, SWL) or DHP. Lignin content was estimated using the LTGA method (ECL: 35%, w/w) or the Klason method (SWL: 28%, w/w). Percentage values of monomers are normalized to the amount of compound **1** (set at 100%) and are shown in parentheses. For dimers, values in parentheses are the mol % of G-G dimers.

Compound	Source			Bond Type
	ECL	SWL	DHP	
		μmol g^{-1}		
Monomers				
G (major); **1**	291 (100)	950 (100)	630 (100)	
G (minor); **2**	17 (6)	50 (5)	31 (5)	
H, **3**	54 (19)	9 (1)	105 (17)	
Coniferyl aldehyde; **4**	11 (4)	39 (4)	25 (4)	
Coniferyl alcohol; **5, 6**	49 (16)	68 (7)	226 (36)	
Total yield	422	1116	1017	
Mol % of dimers relative to **1**	31.5	29	36	
Dimers (G-G)				
7 + 8	23.7 (24)	91.9 (33)	62.2 (17)	5–5
9 + 10	39.4 (40)	83.6 (30)	194.1 (53)	β-5
11 + 12	24.9 (24)	1.9 (traces)	80.5 (22)	β-β
13	9.1 (9)	78.2 (28)	21.9 (6)	β-1
14	2.8 (3)	18.1 (6.5)	11.0 (3)	4-O-5

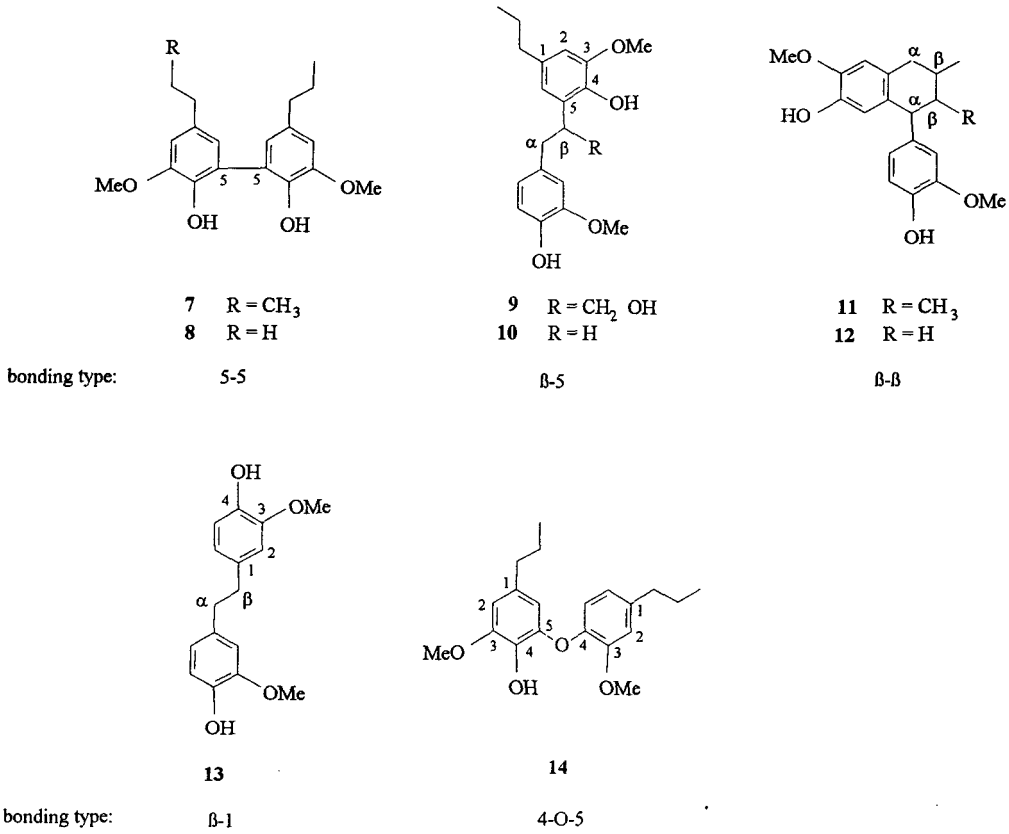

Figure 3. Chemical structures of the main dimer lignin components obtained after thioacidolysis and subsequent desulfurization. OMe, methoxy.

matic acetate protons (2.2 ppm), protons in aromatic methoxyl groups (3.7 ppm), and aromatic protons (6.8–7.1 ppm). Signals from side-chain protons (4.1, 4.2, and 4.7 ppm) were very weak. The Fourier transform IR spectrum of ECL displayed the typical features of a gymnosperm lignin (Faix, 1993) with bands at 1520 (aromatic skeletal vibrations), 1470 (C—H deformation in methoxyl groups), 1330 (G ring condensed), 1270 (G ring), 1230 (C—C, C—O, and C=O stretch in G units), 1150 (C—H in plane in G units), 1030 (C—H in plane in G units), and 830 cm^{-1} (C—H out of plane in H units; weak).

Elemental Analysis

The composition of ECL, as determined by elemental microanalysis, is shown in Table II. Compared to spruce milled wood lignin, obtained by solvent extraction from finely ground spruce wood, the ECL samples (collected d 3, 5, and 6 after elicitation; marked as ECL 3, ECL 5, ECL 6) had a lower carbon and a remarkably higher oxygen content. In this respect, the elemental composition of ECL was very similar to that of the untreated spruce wood. The most striking difference was the significant appearance of nitrogen in ECL (approximately 3.7%). With an average amino acid molecular mass of 120 D (Lehninger, 1970), this nitrogen content corresponds to a protein content of 31.7% (w/w).

Table II. *Elemental composition (%, w/w) of the extracellular polymer from spruce cell cultures (ECL) harvested at d 3, 5, and 6 in comparison with spruce milled wood lignin MWL and untreated spruce wood*

Sample	C	H	O	N
ECL 3	49.6	5.9	41.1	3.5
ECL 5	44.3	6.1	45.8	3.8
ECL 6	44.4	6.1	45.6	4.0
MWL	62.4	5.5	31.2	0.8
Spruce wood	47.3	6.3	45.8	0.6

Amino Acid and Sugar Analysis

The nitrogen content of ECL was not significantly decreased upon digestion with pronase E, trypsin, or chymotrypsin. In view of this protease resistance, a hydrolysis of the extracellular polymer in 6 M HCl was performed to determine the relative amounts of amino acids. ECL was rich in Hyp (26–31%), Pro (11–13%), Ser (9–11%), Lys (7–9%), Gly (7–8%), and Val (6–8%), thus exhibiting similarities to extensin cell wall proteins from primary walls of sycamore (Lamport and Northcote, 1960) as well as from wood of loblolly pine (Bao et al. 1992) (Table III). It contained no uronic acids as measured by the method of Blumenkrantz and Asboe-Hansen (1973). The total amount of neutral sugars was determined by the anthrone method

Table III. *Amino acid composition (mol %) of the extracellular polymer from spruce cell cultures (ECL) harvested at d 3, 5, and 6 after hydrolysis in 6 M HCl, in comparison with purified extensins from Acer pseudoplatanus (Ap) (from Lamport and Northcote, 1960) and Pinus taeda (Pt) (from Bao et al., 1992)*

Amino Acid	Mol % of Amino Acid				
	ECL 3	ECL 5	ECL 6	Extensin (*Ap*)	Extensin (*Pt*)
Hyp	31.4	30.5	26.2	12.9	11.5
Asp/Asn	n.d.	n.d.	1.5	8.5	8.1
Glu/Gln	4.1	4.1	4.8	8.2	3.2
Ser	10.3	10.9	9.3	9.4	6.3
Gly	7.4	7.2	7.7	3.2	7.9
His	2.2	1.9	1.9	3.4	0.8
Arg	1.4	1.1	1.5	3.3	2.2
Thr	4.6	4.6	5.1	3.8	4.8
Ala	4.5	5.0	5.4	3.6	6.4
Pro	12.0	12.6	10.8	4.5	24.0
Tyr	1.2	1.3	2.0	5.1	4.1
Val	6.5	6.5	7.5	6.2	5.7
Ile	2.1	1.2	3.0	3.8	1.3
Leu	2.6	2.2	3.9	5.7	3.0
Phe	1.2	1.5	1.8	4.4	1.5
Lys	8.5	8.6	6.9	13.0	8.5

(Ayers et al. 1976), which yielded a value of approximately 14% (w/w) in all ECL samples. Gal, Glc, and Ara were identified as major sugars after hydrolysis in trifluoroacetic acid and subsequent analysis of the liberated monosaccharides in a sugar analyzer according to the method of Fengel and Wegener (1979): However, the yield of the recovered sugars was very low (<0.25%; w/w) when compared to the high amount of neutral sugars expected based on measurement by the anthrone method.

Analysis of Lignin Structure in Spruce Needles and Hypocotyls

The lignin contents of needles and hypocotyls were 6 and 21%, respectively, of cell-wall dry weight (LTGA method). The yield of the main G thioacidolysis product, product **1** (Fig. 2), from needles and hypocotyls of spruce seedlings was higher (1310 and 1131 μmol/g lignin) than that of SWL (950 μmol/g lignin). The relative yield of H monomers (**3**) from needles and hypocotyls was about 8-fold higher than in the case of SWL. All analytical data are summarized in Table IV.

The proportion of coniferyl aldehyde end groups (**4**) from needles and hypocotyls was comparable to that in SWL and ECL. The amount of coniferyl alcohol end groups (**5** + **6**) in needles was higher in hypocotyls (16%) than in SWL (6 and 7%, respectively) and resembled the value obtained with ECL (16%). The total yields of dimeric thioacidolysis products from spruce needles and hypocotyls

Table IV. *Degradation product yields obtained after thioacidolysis (monomers) and desulfurization (dimers) of needles and hypocotyls from spruce*

Values are expressed as μmol/g lignin. Lignin content (w/w) was estimated by the LTGA method using a DHP as a calibration standard (6% for needles and 21% for hypocotyls). Percentage values of monomers are normalized to the amount of compound **1** (set at 100%) and are shown in parentheses. For dimers, values in parentheses are the mol % of G-G dimers.

Compound	Needles (Seedling)	Hypocotyls (Seedling)	Bond Type
	$\mu mol\ g^{-1}$	$\mu mol\ g^{-1}$	
Monomers			
G (major); **1**	1310 (100)	1131 (100)	
G (minor); **2**	66 (6)	47 (6)	
H; **3**	95 (8)	79 (7)	
Coniferyl aldehyde; **4**	107 (4)	69 (4)	
Coniferyl alcohol; **5, 6**	120 (16)	52 (16)	
Total yield	1698	1379	
Mol % of dimers relative to **1**	27	21	
Dimers (G-G)			
7 + **8**	95.3 (27)	80.9 (28)	5–5
9 + **10**	141.2 (40)	118.5 (41)	β-5
11 + **12**	Traces	Traces	β-β
13	98.9 (28)	69.3 (24)	β-1
14	31.8 (5)	20.2 (7)	4-O-5

(367 and 289 μmol/g lignin) were comparable to the case of SWL (274 μmol/g lignin) and higher than in the case of ECL (100 μmol/g lignin). These values correspond to dimer proportions of 28% (needles) and 26% (hypocotyls) when related to G monomer **1** (ECL 34%, SWL 29%). The cross-linkage types quantitated in Table IV were generally similar to those in SWL, except for the relative amounts of β-5 and 5–5 linkages, which were higher and lower, respectively, in seedlings than in SWL.

DISCUSSION

Lignin Subunit Composition

Cell cultures of *P. abies* (L.) Karst have been shown to respond to treatment with an elicitor preparation from the spruce needle pathogen *R. kalkhoffii* with the excretion of a lignin-like material (Meßner and Boll, 1993). In the present study, the chemical structure of the released material has been investigated in some detail. The purification and cDNA cloning of the lignin biosynthetic enzyme, CAD, from the same spruce cell culture have previously been described. In addition, both CAD enzyme activity and CAD transcript level were shown to be induced by elicitor in the cultured spruce cells and by ozone in spruce seedlings (Galliano et al., 1993a, 1993b).

The structural evidence obtained here reveals significant differences between the extracellular cell culture lignin (ECL) and SWL. The low yield of monomeric thioacidolysis products and the higher relative proportion of thioacidolysis dimers indicate that ECL is a highly branched polymer with a large amount of interunit C-C bonds. The high amounts of H units suggest that the biosynthetic process is similar to the formation of gymnosperm compression wood and middle lamella lignins (Fukushima and Terashima, 1991a, 1991b). Microanalytical and autoradiographic studies have indicated that the deposition of H units typifies the early developmental stages of lignification occurring in the middle lamella (Whiting and Goring, 1982; Sorvari et al., 1986; Terashima and Fukushima, 1988; Fukushima and Terashima, 1990). In all cases in which it has been examined, an elevated content of H units has also been observed for lignins formed de novo in cell culture systems (*Glycine*: Nimz et al., 1975; *Rosa*: Mollard and Robert, 1983; *Pinus*: Fukuda et al., 1988, Campbell and Ellis, 1992, Eberhardt et al., 1993; *Picea*: Brunow et al., 1993; *Camellia*: Zaprometov et al., 1993), and it has frequently been observed in intact plants challenged with pathogens (*Raphanus*: Asada and Matsumoto, 1972; *Triticum*: Ride, 1975; *Cucurbita* sp.: Hammerschmidt et al., 1985; *Prunus*: Doster and Bostock, 1988; *Cucumis*: Robertsen and Svalheim, 1990). Some of these studies have used nitrobenzene or cupric oxide oxidation. These procedures can misidentify lignin H units when Tyr- (e.g. tyramine) or *p*-coumaroyl-derived substituents are present, because these phenolics may give rise to the same oxidation product (Lapierre, 1993). However, increased lignin H units have been detected in some of the studies by the more specific method of thioacidolysis. Literature reports of the H content of various conifer lignins are summarized in Table V.

The present results concerning an elicitor-induced spruce lignin reveal considerable similarities to early developmental lignins. These comprise all cell culture lignins of Table V, regardless of whether they were formed in response to a special composition of the culture medium or to elicitor treatment. The analogy, in particular, extends to the lignin material produced by spruce cell cultures without the use of elicitor (Brunow et al., 1990, 1993).

The regulatory process responsible for the suppression of precursor 3-hydroxylation and for the preferred incorporation of *p*-coumaryl alcohol into newly formed lignins is as yet unknown. In general terms, a rapid lignin induction is likely to exhaust the cellular pool of coniferyl alcohol and its precursor pools, such as feruloyl-CoA. The pool of *p*-coumaroyl-CoA may be utilized instead, since the lignin-related spruce enzymes, hydroxycinnamoyl-CoA ligase (Lüderitz et al., 1982) and reductase (Lüderitz and Grisebach, 1981), as well as CAD (Lüderitz and Grisebach, 1981; Galliano et al., 1993b), are all known to accept the p-coumaroyl-derived substrates to some extent. This pool depletion hypothesis is consistent with the general occurrence of increased H units in all gymnosperm and angiosperm de novo lignins studied, regardless of how they were induced (by developmental or stress signals). Methanol extraction of the cultured cells and HPLC showed that the steady-state pools of coniferyl and *p*-coumaryl alcohols, as well as their β-D-glucosides, were extremely small (data not shown). In addition, regulatory effects may exist at the level of enzyme activities (in particular the 3-hydroxylase reaction) rather than substrate pools.

Lignin Interunit Linkages

The composition of the dimer fraction after thioacidolysis has revealed further differences between ECL and SWL. Compared to SWL, a much higher content of dimers derived from pinoresinol structures and approximately one-third of the amount of diarylpropane structural units were detected in ECL. Similar results have previously been obtained by thioacidolysis of synthetic lignins (DHPs; Tollier et al., 1991). On the other hand, clear differences were also observed between ECL and DHP, e.g. a higher amount of biphenyl-derived structures and a lower yield in β-5 dimers in ECL. Therefore, one can conclude that the composition of interunit linkages in the ECL are intermediate between those of gymnosperm wood lignins and the in vitro lignins. The low yield of monomeric thioacidolysis products and the high relative amount of dimeric products compared to SWL point to ECL as being a highly condensed polymer with a large proportion of interunit C-C bonds. This feature has again also been reported for lignins of compression wood and of the cell-wall middle lamella (Eom et al., 1989; Fukushima and Terashima, 1990). In analogy to the proposal made for compression wood lignin, the present extracellular stress lignin could originate from an analog of the "Zulaufverfahren" proceeding with high local monolignol concentrations (Higuchi, 1985). This mechanism, which may involve vesicles (Sandermann, 1994), has also been proposed for a developmental lignin excreted by spruce cell cultures (Brunow et al., 1993). The

Table V. *Relative content of H units of various gymnosperm lignins*

The plant species and the analytical methods used are indicated. Most of the listed lignins were formed in response to growth conditions. In two cases, stress lignins induced by fungal elicitors were studied. el., Elevated but not quantified. OMe, methoxy.

Plant Species	Relative Yield of H	Method of H Unit Quantification	References
	% *of G*		
Wood lignins			
P. abies wood	1–2	Nitrobenzene oxidation	Sarkanen and Hergert (1972)
P. abies wood	1–2	Thioacidolysis	Lapierre (1993)
P. sylvestris wood	1–2	Nitrobenzene oxidation	Sarkanen and Hergert (1972)
P. pinaster wood	2	Thioacidolysis	Lapierre (1993)
P. abies compression wood	15	Nitrobenzene oxidation	Westermark (1985)
Pinus thumbergii compression wood	18	Nitrobenzene oxidation	Fukushima and Terashima (1991a)
P. pinaster compression wood	21	Thioacidolysis	Lapierre (1993)
Ginkgo biloba compression wood	9	Nitrobenzene oxidation	Fukushima and Terashima (1991b)
Needle and hypocotyl lignins			
G. biloba leaves	10	$KMnO_4$-$NaIO_4$ oxidation	Miksche and Yasuda (1977)
P. abies needles	6	$KMnO_4$-$NaIO_4$ oxidation	Miksche and Yasuda (1977)
P. sylvestris needles	7	$KMnO_4$-$NaIO_4$ oxidation	Miksche and Yasuda (1977)
P. abies needles (from seedlings)	9	Thioacidolysis	This work
P. abies hypocotyls (from seedlings)	9	Thioacidolysis	This work
Middle lamella lignins			
Picea mariana	40% less OMe	IR spectroscopy, pyrolysis	Whiting and Goring (1982)
P. abies	6	Nitrobenzene oxidation	Sovari et al. (1986)
P. thumbergii	el.	Microautoradiography	Terashima and Fukushima (1988)
G. biloba	el.	Microautoradiography	Fukushima and Terashima (1991b)
Cell culture lignins			
P. taeda (cell walls)	19	Nitrobenzene oxidation	Fukuda et al. (1988)
Pinus banksiana (cell walls), elicitor	36	Nitrobenzene oxidation	Campbell and Ellis (1992)
P. taeda (cell walls)	17	Thioacidolysis	Eberhardt et al. (1993)
P. abies (extracellular)	10; 14	Thioacidolysis	Brunow et al. (1993)
P. abies (extracellular), elicitor	19	Thioacidolysis	This work

present results leave open whether fungal elicitor induced de novo lignin biosynthesis or only induced ECL transport. The composition of interunit linkages in spruce seedlings was not similar to that of mature wood (see Table IV). This confirms that lignins in differentiating tissues differ structurally from lignins in mature wood.

Lignin Protein Component

The protein component of ECL (about 32%, w/w) has a high content of Pro and Hyp and therefore resembles extensins (Hyp-rich glycoproteins). Extensins are well-known as a constitutive structural component of primary cell walls (Lamport and Northcote, 1960; Whitmore, 1982) and of conifer wood (Bao et al., 1992). In addition, extensins have been shown to be induced during phytopathological defense reactions (Showalter, 1993) and ozone treatment (Schneiderbauer and Sandermann, 1990). Extensins are believed to be insolubilized in the cell wall during host-pathogen interactions by a peroxidase/H_2O_2-mediated cross-linking process (Bradley et al., 1992). Whitmore (1978) has suggested the bonding of lignin precursors to Hyp-containing proteins by peroxidase catalysis. In an early report, an ECL/extensin complex excreted by cultured soybean (*Glycine max*) cells was described (Moore, 1973). The presence of carbohydrate residues in ECL (about 14%, w/w) is consistent with the general occurrence of lignin-carbohydrate cross-linkages in cell walls (Iiyama et al., 1994). Cellulose fibrils were absent from ECL as shown by microscopy under polarized light (data not shown).

In summary, the extracellular product analyzed here can be termed "stress lignin" on the following bases: (a) absolute elicitor dependence for its formation; (b) presence of an Hyp-rich protein component. Such proteins are known to be stress-related (Showalter, 1993); and (c) distinct features of lignin structure (H units, cross-linkages). Still, the chemical structure of ECL is not unique but resembles that of special developmental gymnosperm lignins, such as of compression wood, middle lamellae, and induced cell cultures. Elicitor appeared to act like a regulatory signal for early lignin formation but at the "wrong" (abnormal) time. The induction of CAD by fungal elicitor and ozone, respectively, also appears to be related to normal developmental processes because spruce was found to contain only a single CAD gene and a single CAD isoenzyme form (Galliano et al., 1993a, 1993b).

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